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The Role of Shell Granules and Accessory Cells in Eggshell Formation in *Convoluta pulchra* (Turbellaria, Acoela)

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Abstract. Most turbellarian embryos are surrounded by a sclerotinized eggshell originating from polyphenol-containing eggshell-forming granules (EFGs). Although embryos of the acoel *Convoluta pulchra* are surrounded by a shell, it is not sclerotinized. Therefore, in the absence of polyphenols as a marker for EFGs, it was not clear which, if any, of the granules of the oocyte function in eggshell synthesis. In this study, electron-opaque, elliptical granules with a characteristic frothy component and a diameter of 480 nm were identified in the oocyte as EFGs by their participation in eggshell formation. In addition, it was shown that accessory cells to the oocyte initiate synthesis of the shell by producing a thin, granular, electron-opaque primary shell, against which the contents of the EFGs are released by exocytosis. Morphological components of the shell and stages of its synthesis are described. A second type of membrane-bound granule and the lipid droplets that occur in the ooplasm were found not to be involved in eggshell formation and are probable sources of nutrients for the developing embryo. Possible implications of the findings for taxonomy and phylogeny are discussed.

Introduction

The zygotes of acoel turbellarians, like those of all platyhelminthes studied to date, are enclosed in a shell following fertilization (see Rieger *et al.*, 1991, for Turbellaria; Fried and Haseeb, 1991, and Coil, 1991, for parasitic platyhelminths). In all cases so far examined, the eggshell appears to arise from eggshell-forming granules (EFGs) exocytosed from one of the cells that ultimately comes to lie within the shell (oocytes in the entolecithal archoophorans, yolk cells in the ectolecithal neoophorans and parasitic platyhelminths). Among archoophoran turbellarians, EFGs have been described by transmission electron microscopy (TEM) from the oocytes of at least one member of most orders (*Polycladida:* Boyer, 1972; Domenici *et al.*, 1975; Gammon, 1979; Ishida *et al.*, 1981; Espinosa, 1986; Ishida and Teshirogi, 1986; *Macrostomida:* Gremigni *et al.*, 1987; Kucera, 1987; *Acoela:* Gremigni, 1988; Smith *et al.*, 1988; *Nemertodermatida:* Smith *et al.*, 1988). Relatively few TEM studies of archoophorans, however, have examined formation of the eggshell (see Ishida, 1989; Falleni and Gremigni, 1989).

Whereas EFGs in most platyhelminths examined to date contain polyphenols, they have not been found in the oocytes of the Acoela (Thomas *et al.*, 1985; Chandler and Thomas, 1986, 1987; Gremigni, 1988; Smith *et al.*, 1988; Falleni and Gremigni, 1989, 1990) or the Nemertodermatida (Thomas *et al.*, 1985; Smith *et al.*, 1988), the presumably primitive turbellarian orders that constitute the Acoelomorpha. In the acoels, the shell is proteinaceous and non-sclerotinized (Falleni and Gremigni, 1989). Because the eggshells of all other non-acoelomorphan platyhelminths studied so far appear to be sclerotinized, the process of eggshell formation in the acoels merits further study. For example, in the absence of the polyphenolic marker, it is difficult to ascertain with certainty which, if any, of the several types of granules in the oocyte give rise to the eggshell. Falleni and Gremigni (1989) have implicated a population of electron-opaque granules with a diameter of 0.4–0.5 μm as EFGs in the oocytes of the acoel "*Convoluta psammophyla*" (? = *Pae-*

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domecynostomum psammophilum Beklemischev, 1957), but have not examined the mechanism by which these granules contribute to the formation of the shell. Although it seems likely that exocytosis of the granules, which occurs during eggshell formation in other turbellarians, is involved in production of the eggshell of the acoels (Falleni and Gremigni, 1990), that is not clear from the published studies.

Also unclear is the question of whether cells other than the oocyte are involved in synthesis of the eggshell in acoels, as they are in other turbellarians (*e.g.*, Giesa, 1966; Bunke, 1982; Ishida, 1989). Mature oocytes in both acoels and nemertodermatids are nearly always surrounded by "follicle" or "accessory" cells, whose function has not been elucidated, although it is usually suggested that they are responsible for heterosynthetic yolk production or that they assist in the production of the eggshell (see Rieger *et al.*, 1991).

The present study examines eggshell formation in the acoel *Convoluta pulchra* with particular attention to the following questions: (1) do any of the granules of the oocyte participate in the formation of the eggshell; (2) if so, what is the mechanism by which they participate; (3) are other cell types involved in eggshell synthesis; and (4) what is the morphology of the shell itself? A preliminary report of these findings has been presented elsewhere (Chandler *et al.*, 1988).

Materials and Methods

Experimental organism

Convoluta pulchra (Family Convolutidae; Smith and Bush, 1991) was extracted according to the methods of Hulings and Gray (1974) from sediment samples collected at mid-tide level at a coastal inlet near Fort Fisher, North Carolina.

Procedures for preliminary observations

To determine the time course of egg-laying in *Convoluta pulchra*, gravid acoels were isolated in pairs in wells of Falcon® 96-well plates. The individual cultures were maintained in Millipore®-filtered seawater (MFSW) at a constant temperature of 20°C and a light:dark cycle of 16:8 h. The worms were monitored closely to determine if the acoels lay their eggs according to a diurnal pattern. Under these conditions, worms began egg-laying within approximately 1 to 1.5 h after exposure to light.

Experimental design

Ten pairs of acoels with large eggs were placed in Falcon® plates. The animals were placed into darkness at 9:30 p.m. and returned to light at 6:30 a.m. Worms were fixed for electron microscopy every half hour, from 7:00 a.m. until 11:30 a.m. Other worms were allowed to lay eggs, which were fixed for electron microscopy.

Procedures for electron microscopy

Adult worms and laid eggs were fixed in 1% glutaraldehyde, 4% paraformaldehyde, 0.1 *M* HEPES buffer (pH 7.4), 1 m*M* $CaCl_2$, and 10% sucrose [modified from McDowell and Trump (1976)], rinsed in buffer, post-fixed in HEPES-buffered 1% OsO_4, and embedded in Spurr's low viscosity epoxy resin (Spurr, 1969). Ultrathin sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Philips 201C transmission electron microscope.

Procedures for morphometric analysis

The Feret diameters (see Weibel, 1979) of the three types of granules were measured with a Zeiss ZIDAS digitizer. The Feret diameter of each granule profile was measured between two lines parallel to the long axis of the photographic print. The most mature stage of development in which all types of granules were present (Primary Shell Synthesis Stage, described below) was chosen for measurement. To avoid measuring the same granule more than once, 1.8 μm separated the thin sections measured and non-overlapping micrographs were taken from each thin section examined. Approximately 200 granules from electron micrographs magnified 28,000× were measured and the size-frequency distribution of Feret diameters for granule Types A and B were plotted. For the Type A granules, the distribution was corrected for profiles overlooked in the smallest categories as described in Weibel (1979). The actual diameter of Type A granules (D) was estimated from the average Feret diameter (d) using the relationship $D \cong \frac{4}{\pi} \cdot d$ (Weibel, 1979).

Oocytes at different stages of shell maturation were analyzed to determine the volume densities (V_v; % of oocyte volume occupied by granules) of Types A and B granules and lipid droplets. Non-overlapping micrographs along two right-angled transects were taken from the germinal vesicle to the oolemma. Volume densities were determined by point-count analysis, using the oocyte as the reference space (Weibel, 1979). To determine whether these volume densities changed in the oocyte as development of the shell proceeded, the volume densities were arcsin-square root transformed and subjected to ANOVA Planned Comparison.

The embryos contained within laid eggs fixed for electron microscopy were observed to be separated from the inner edge of their shells. This could occur if the shell swells and lifts away from the embryo or if the embryo

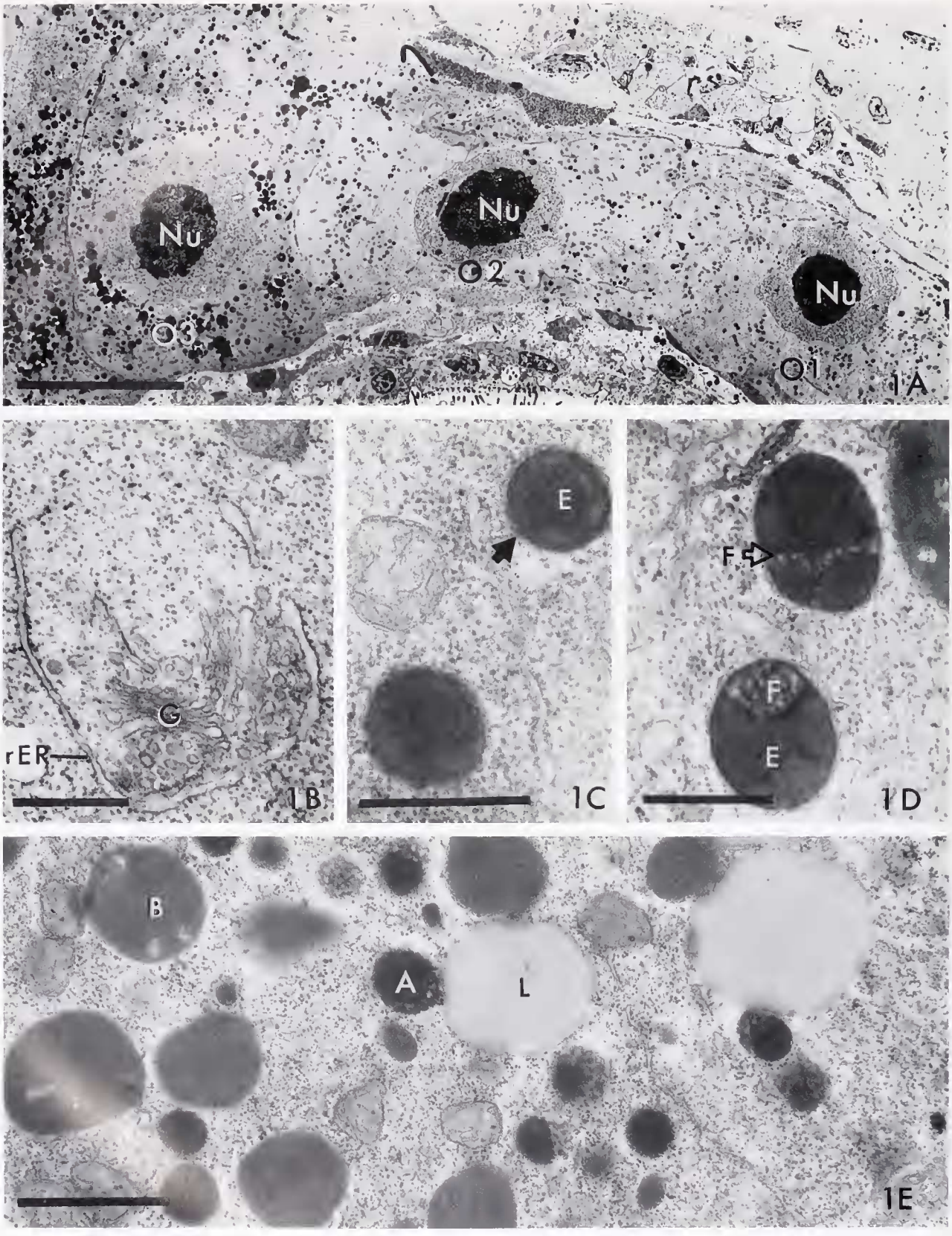

Figure 1. (A) Overview of a column of maturing oocytes showing the nuclei (germinal vesicles) with large nucleoli (Nu) and the increase in the number of granules in the ooplasm with development. O1 = oocyte synthesizing Type A granules only; O2 = oocyte synthesizing Types A and B granules; O3 = oocyte containing Type A and B granules and lipid droplets. Bar = 15.0 μm. (B) Cisternae of rough endoplasmic

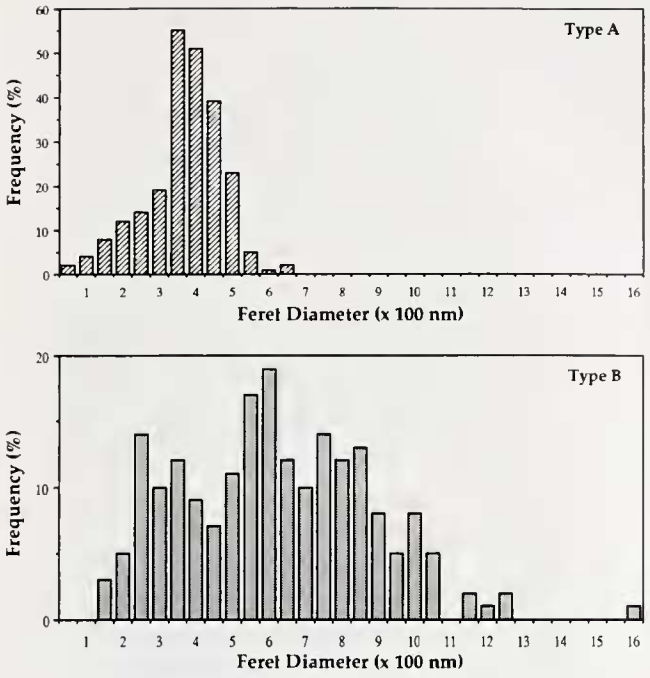

Figure 2. Feret diameter distribution of granule Types A and B. Each number on the abscissa represents the stated value ±0.5; n = 200.

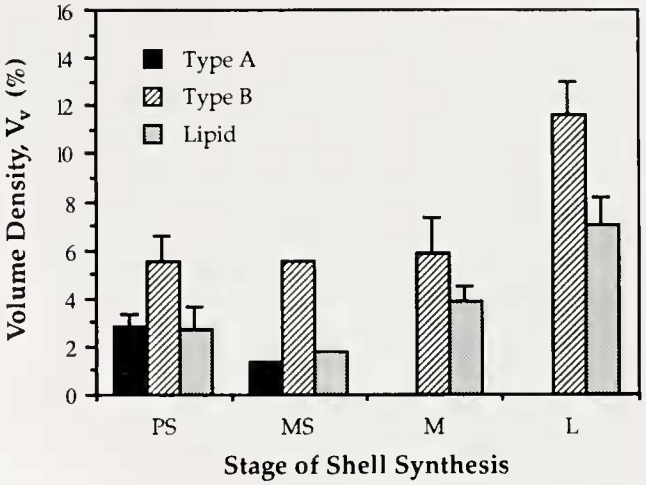

Figure 3. Volume densities of granular inclusions. PS = stage of Primary Shell synthesis; MS = stage of Mature Shell synthesis; M = stage of Mature Shell; L = stage of Laid Shell.

loses some of its volume, shrinking away from the shell. Since the two possibilities affect the interpretation of the changes in volume densities of the granules, the absolute volumes of eggs prior to egg-laying and of embryos after egg-laying were determined. Serial 2 μm-thick sections were viewed with a Zeiss Axioskop light microscope equipped with a Sony DXC-3000A color video camera and a Sony PVM-1343MD Trinitron color monitor. Each section of egg or embryo was traced from the monitor screen onto transparent plastic. The ZIDAS was used to calculate the area of each drawn section; the area, in turn, was multiplied by the thickness of the sections and these numbers summed for all sections to determine the volume of each egg or embryo. Four eggs with mature shells and four laid eggs were analyzed. ANOVA was used to compare the mean volume of eggs with mature shells to that of embryos within the laid eggs.

Results

Stages of shell formation

Based on the ultrastructural features described in detail below, development of the eggshell in *Convoluta pulchra* was divided into four sequential stages: Primary Shell Synthesis, Mature Shell Synthesis, Mature Shell, and Laid Shell. The germinal vesicle persists into the Mature Shell stage, during which it breaks down. Because the time of fertilization is not known for the species, the Mature Shell stage may be an oocyte or a zygote, and will therefore be referred to as the "egg" (unfertilized or fertilized). At the Laid Shell stage, embryos are developing.

Description of granules and lipid droplets

Granules we have termed Type A granules were the first to appear in developing oocytes (Fig. 1A, egg 1). Oocytes at this stage had a large germinal vesicle containing euchromatin and a single prominent nucleolus. Long strands of rER occurred in the cytoplasm. Additional rER and Golgi were often found close to one another, usually with the rER almost encircling the Golgi in a horseshoe configuration (Fig. 1B). Mitochondria were abundant. The forming Type A granules were spherical to ellipsoidal and electron-opaque with slightly lucent internal areas (Fig. 1C); profiles of the granules did not exceed 330 nm in early oocytes.

In more mature primary oocytes, the Type A granules were more complex, larger, and more abundant. Mature Type A granules consisted of two components, electron-

reticulum (rER) almost totally encircling the Golgi (G) in an oocyte synthesizing Types A and B granules. Bar = 0.5 μm. (C) An immature Type A granule before the frothy element is evident. Only the electron-opaque component (E) is present. The membrane surrounding the granule is indicated by the arrow. Bar = 0.5 μm. (D) Two mature Type A granules with their typically elliptical profile. Both the electron-opaque component (E) and the frothy element (F) can be distinguished. Bar = 0.5 μm. (E) A field of granules from an oocyte slightly older than oocyte 3 in Figure 1. Type A granules (A), Type B granules (B), and lipid droplets (L) occur in the cytoplasm. Bar = 1.0 μm.

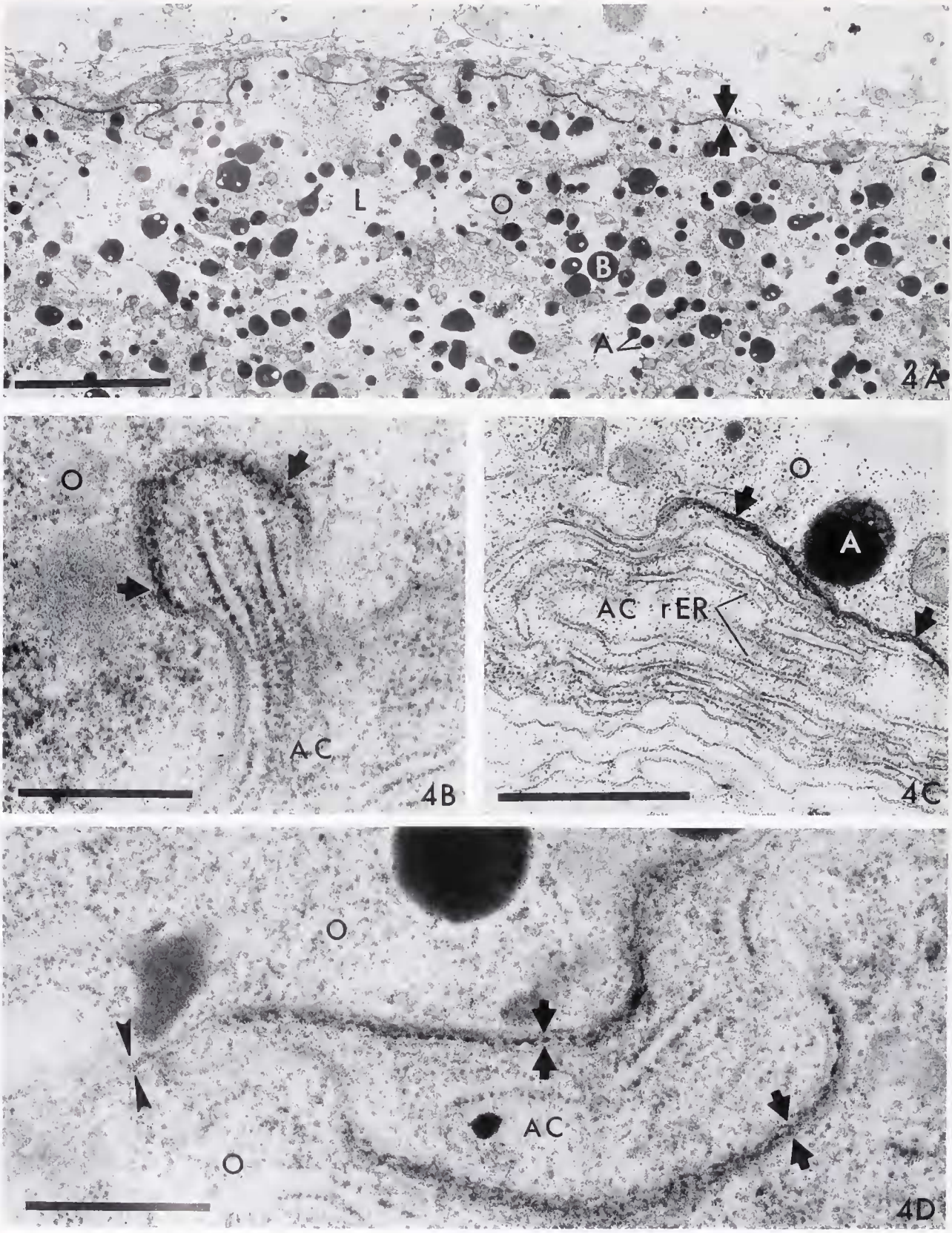

Figure 4. (A) An oocyte (O) with a primary shell (arrows). Type A granules (A), Type B granules (B), and lipid droplets (L) can be identified in the ooplasm. Bar = 5.0 μm. (B) A region of the surface of an oocyte (O) in contact with an accessory cell (AC). In the part of the region of contact indicated by the arrows the granular primary shell has formed. Bar = 1.0 μm. (C) An accessory cell (AC) with an extensive rough

opaque globules and a frothy material that often formed a cap at one pole of the granule (Fig. 1D, 1E). The mature Type A granules from an oocyte in the Primary Shell Synthesis stage exhibited an average Feret diameter of 380 nm (n = 199), giving an estimated average diameter of 480 nm; the largest profile measurement was 640 nm (Fig. 2). Type A granules reached an average volume density of 2.9% in Primary Shell Synthesis stage oocytes (Fig. 3). Although Type A granules were not observed to marginate at the time of eggshell formation, their volume density dropped to 1.4% in the single Mature Shell Synthesis stage oocyte observed, and was zero in both Mature Shell and Laid Shell stages (Fig. 3).

A second type of granule, termed the Type B granule, was first observed in oocytes that still possessed a large germinal vesicle (Fig. 1A, egg 2). More Golgi bodies and rER occurred in these oocytes than in those synthesizing only Type A granules. Mature Type B granules were irregular spheres with a flattened edge (Fig. 1E). The Type B granules were less electron-opaque than the Type A granules and contained internal electron-lucent patches. The mature Type B granules from a Primary Shell Synthesis stage oocyte exhibited an average Feret diameter of 590 nm (Fig. 2); the largest measurement was 1570 nm (n = 200). Calculation of an average diameter from the Feret diameter was not attempted because Type B granules appeared to depart significantly from a spherical shape. The volume density of Type B granules was 5.5% in Primary Shell Synthesis stage oocytes as well as in the single Mature Shell Synthesis stage oocyte, 5.9% in Mature Shell stage oocytes, and 11.6% in Laid Shell stage embryos (Fig. 3). The volume densities of Type B granules were not statistically different during shell deposition (comparing Primary Shell Synthesis stage oocytes to Mature Shell stage oocytes: $F = 0.004$, $df = 1, 10$); however, the increase seen in laid eggs was significant ($F = 11.08$, $df = 1, 10$; $P < .05$).

Lipid droplets (Fig. 1E) appeared in oocytes that had begun synthesis of Type B granules, but were still in the germinal vesicle stage (Fig. 1A, egg 3). Lipid droplets appeared to have no membrane. The volume density of lipid droplets was 2.7% in Primary Shell Synthesis stage oocytes, 1.8% in the single Mature Shell Synthesis stage oocyte, 3.9% in Mature Shell stage oocytes, and 7.0% in laid eggs (Fig. 3). As was the case for the Type B granules, the volume density of lipid droplets was statistically constant during shell synthesis ($F = 0.22$, $df = 1, 10$), but increased significantly in laid eggs ($F = 9.13$, $df = 1, 10$; $P < .05$).

Morphology of eggshell deposition

Primary Shell Synthesis stage. Synthesis of the shell began before the germinal vesicle had broken down. At this stage, both Type A and Type B granules were dispersed throughout the ooplasm, and the oocyte was surrounded wholly or in part by an accessory cell. Electron-opaque material of finely granular composition appeared outside the oocyte, along the irregular contours of the oolemma (Figs. 4A, 4B). This layer was discontinuous in the youngest oocytes of this stage examined, and was only observed where the accessory follicle cell, laden with rER (Figs. 4C, 4D), was in contact with the oolemma. In slightly later stages, this thin primary shell covered the entire oocyte as a layer approximately 50 nm thick.

Mature Shell Synthesis stage. In the single oocyte of this stage encountered, numerous examples of exocytosis of Type A granules were observed (Fig. 5A). Both the homogeneous electron-opaque and the frothy components appeared to be extruded from the cell. Fused granules often produced elongated channels or sacs, the membranes of which were continuous with the plasmalemma (Fig. 5A).

Mature Shell stage. Released Type A granules apparently produced a homogeneous, electron-opaque layer 100–525 nm thick immediately underneath the granular, exogenously originated primary shell, as well as an inner fibrillar network with a thickness of 10–315 nm (Fig. 5B). Clear peripheral vesicles, presumably the remnants of the Type A granules, were visible shortly after exocytosis (Fig. 5B). During the process of exocytosis of the Type A granules, small membrane-bounded fragments of cortical cytoplasm appeared to have been lost from the oocyte (Figs. 5A, 5B).

In somewhat older eggs, a new layer of the shell was observed (Fig. 5C). This thin layer was sandwiched between the granular primary shell and the homogeneous layer produced by the Type A granules and appeared as a stripe that was more electron-opaque than the homogeneous layer. The fibrillar layer was still present just outside the plasmalemma. At this stage, no Type A granules were apparent in the cytoplasm (Fig. 5D).

Laid Shell stage. Laid eggs were encapsulated in a clear, flexible, yet sturdy shell (Fig. 6A). In the laid eggshell, the

endoplasmic reticulum (rER) in contact with an oocyte (O). Arrows indicate the primary shell that has formed along part of the region of contact between the accessory cell and the oocyte. A Type A granule (A) lies close to the region of formation of the thin shell, but exocytosis has not begun at this stage. Bar = 0.5 μm. (D) A region of the plasmalemma of an oocyte (O) with (arrows) and without (darts) an adjacent accessory cell (AC). Note that the primary shell (arrows) is present only where there is an accessory cell. Bar = 0.5 μm.

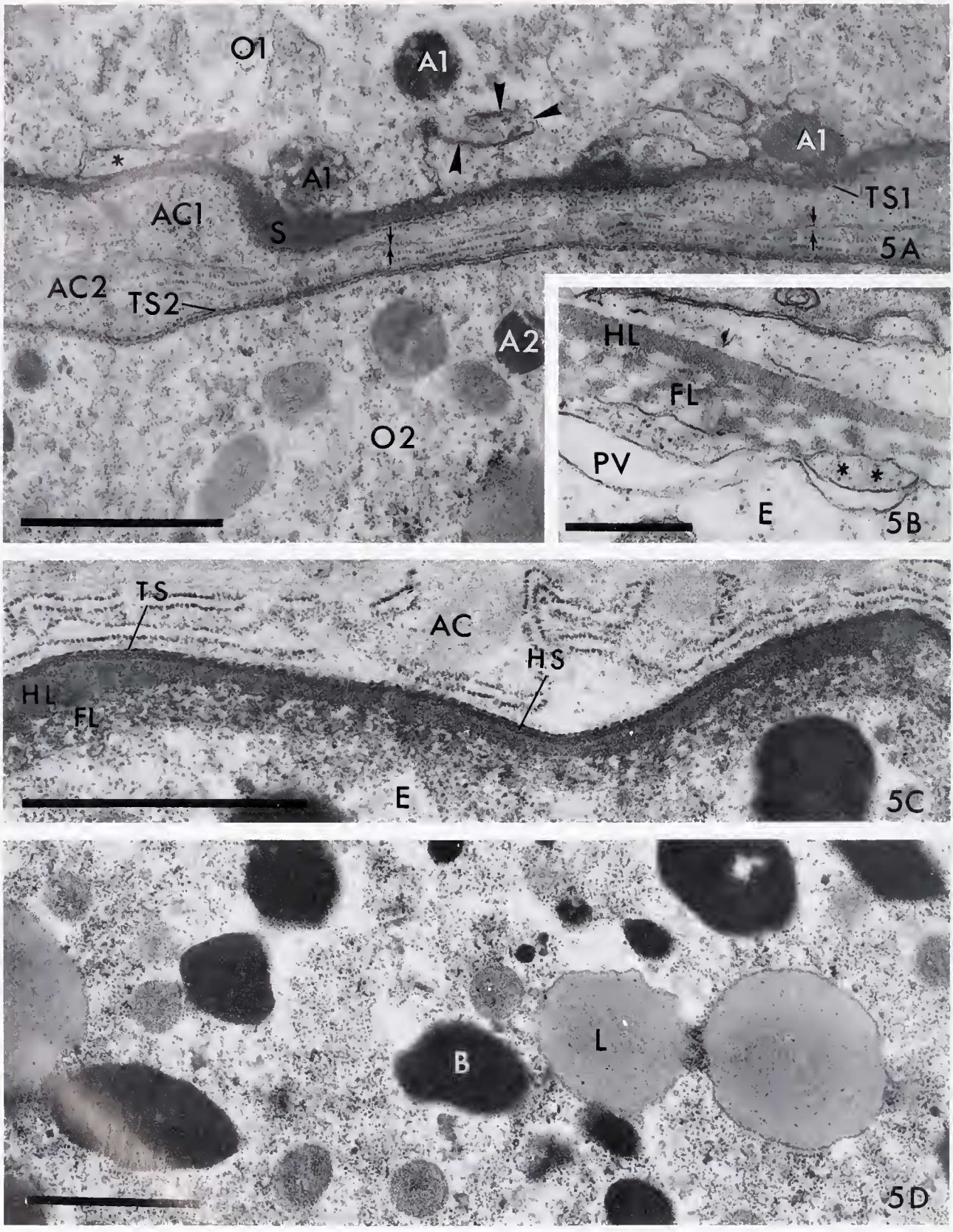

Figure 5. (A) Exocytosis of Type A granules. The contents of Type A granules (A1) are released from the more mature oocyte (O1) to form the layers of its mature shell (S) interior to the thin primary shell (TS1). Darts indicate the long, often tortuous profiles of the membranes of the Type A granules during exocytosis. The asterisk marks a small membrane-bounded fragment of cytoplasm that may no longer be

fibrillar layer could be distinguished, but the granular primary shell, the homogeneous layer, and the stripe could no longer be differentiated; instead, a single, thick, electron-opaque layer occurred peripheral to the fibrillar layer (Fig. 6B). The thickness of the shell was far more homogeneous than that of the forming shell (Fig. 6B). There was a space between the embryo and the shell (Fig. 6A–C). The juvenile worm hatched one day after egg-laying. Before hatching, the worm moved vigorously until the shell gave way.

The mean volume of a Mature Shell stage egg was 325×10^3 μm^3 (n = 4), whereas the mean volume of an embryo after deposition was 180×10^3 μm^3 (n = 4). The volume of the embryo was therefore significantly smaller than that of the unlaid egg ($F = 33.6$, $df = 1, 6$; $P < 0.005$).

Discussion

Eggshell synthesis

Our study demonstrates that the first element of the eggshell in *Convoluta pulchra* is deposited by accessory cells before the Type A granules undergo exocytosis. Accessory cells, laden with rER, appear to envelop an oocyte at the time of primary shell synthesis. This is evidenced by the observation that the thin, granular, primary shell layer appears outside the oolemma in areas of the oocyte surface abutting an accessory cell, whereas regions that do not abut an accessory cell are not covered by primary shell. A role in production of the shell was previously hypothesized for the accessory cell, but not demonstrated (Falleni and Gremigni, 1990). The mechanism by which the material that composes the thin shell is released from the accessory cell is not known. Exocytotic vesicles have not been identified. It is possible that vesicles simply have not been detected, perhaps because they are small, present in low numbers, or do not accumulate in the cytoplasm of the accessory cell. Alternatively, the primary shell may be a product of a reaction between substances located on the surface of the surrounding accessory cell and on the surface of the oocyte. Clearly, the material appears only where the two cells are in contact.

Functionally, the Type A granules of *C. pulchra* are comparable to EFGs of other turbellarians. They undergo exocytosis at the Mature Shell Synthesis stage and are absent from the egg at the end of the Mature Shell stage, indicating that they participate in eggshell deposition.

After the formation of the primary shell, the contents of the Type A granules released by exocytosis become packed against the thin shell made earlier, eventually forming a homogenous layer beneath the primary shell layer. This homogeneous layer probably comes from the electron-opaque portion of the Type A granules. The mature shell enclosing the unlaid egg is characterized by a fibrillar network that forms the innermost layer. The flocculent portion of the Type A granules most likely produces this fibrillar network. These conclusions are based on morphological observations; the detailed cytochemistry of Type A granules is unknown. Also characteristic of the mature shell is an electron-opaque stripe between the primary shell layer and the homogeneous layer. This could represent a zone of reaction between the granular primary shell layer formed by the accessory cell and the homogeneous layer formed from the Type A granule, perhaps associated with some as yet unidentified hardening process. This is suggested by the observation that in the shell of the laid egg, the three outermost layers are no longer discrete. This would be predicted if a reaction between the primary shell and the homogeneous layer produced the stripe and that reaction proceeded until, in the laid eggshell, the stripe replaced the two original layers.

Although the wide variation in the thickness of the components of the shell during early stages of synthesis may be related in part to plane of section, it more likely reflects the number of Type A granules released in a given area. Because the contents of the Type A granules do not maintain their integrity at exocytosis, the contents must be fluid; it is therefore hypothesized that the components

continuous with the egg surface. Two accessory cells (AC1 and AC2), the plasma membranes of which are indicated by arrows, separate the more mature oocyte from a less mature one (O2), which has a thin primary shell (TS2) but has not begun exocytosis of Type A granules (A2). Bar = 1.0 μm. (B) The cortex of the egg after shell formation. Clear peripheral vesicles (PV) occur at the surface of the egg (E) after the homogeneous layer (HL) and fibrillar layer (FL) of the shell have formed around it. The vesicles communicate with extracellular space. Asterisks mark fragments of cortical cytoplasm that may have been cut off from the cytoplasm of the egg. Bar = 0.5 μm. (C) The mature shell of a prelaid egg. The egg (E) is surrounded by its accessory cell (AC) and the thin primary shell (TS). Beneath the primary shell the homogeneous layer (HL) and fibrillar layer (FL) of the mature shell encompass the egg. A homogeneous stripe (HS) can be seen between the primary shell and the homogeneous layer. Although the plasmalemma of the egg is not clearly seen here, adjacent micrographs show that it lies just interior to the fibrillar layer of the shell. Bar = 1.0 μm. (D) A representative section of the ooplasm of the egg after shell formation but before egg-laying. Type B granules (B) and lipid droplets (L) are present, but Type A granules cannot be detected. Compare Figure 5D with Figure 1E, an area of similar size at a stage before formation of the mature shell. Bar = 1.0 μm.

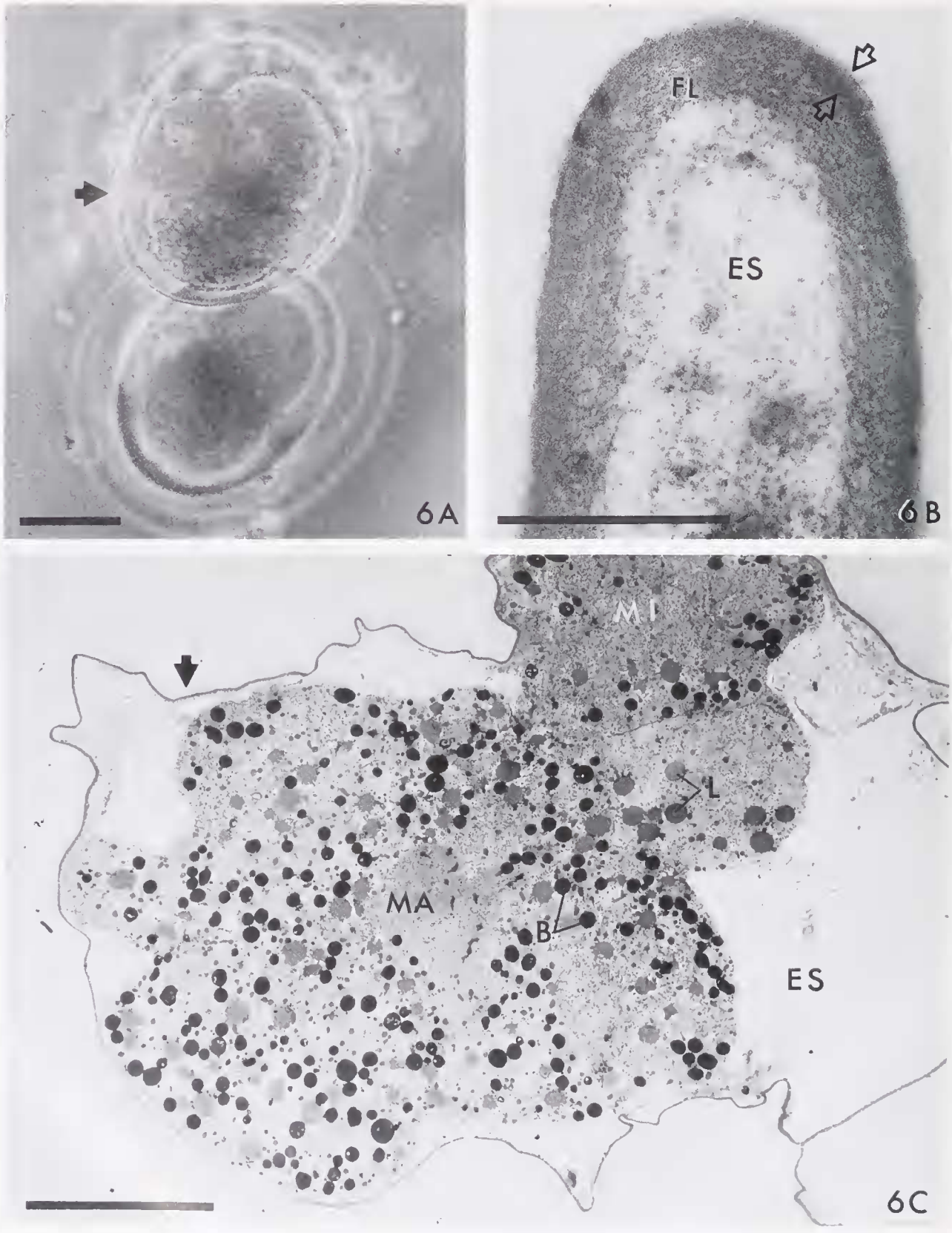

Figure 6. (A) Photomicrograph of two cleaving embryos surrounded by a still flexible, clear eggshell (arrow). Bar = 10.0 μm. (B) A magnified region of the eggshell of a laid embryo. Arrows indicate the layer of the laid shell that probably corresponds to the primary shell, homogeneous stripe, and homogeneous layer; these are no longer discrete. The fibrillar layer (FL) can still be discerned. The thickness of the shell

flow to fill evenly the space between the primary shell and the oolemma before hardening. The shell surrounding the laid embryo shows little variation in thickness.

Because Type A granules are released by exocytosis and profiles in which the membrane of the Type A granule is continuous with the plasmalemma are common, it is likely that the plasma membrane of the egg following shell formation is a mosaic of the original oocyte membrane and the membrane of the Type A granules. The occasional fusion of Type A granules with one another rather than with the plasmalemma could explain what appear to be membrane-bounded fragments of cytoplasm that can sometimes be found between the plasma membrane of the egg and the newly synthesized shell. Because serial sections were not taken, however, it is possible that the apparent fragments are connected to the ooplasm in some plane. The membranes of the clear peripheral vesicles associated with the plasmalemma soon after exocytosis of the Type A granules are hypothesized to be the membranes of the empty Type A granules.

The Type B granules were not observed to participate in eggshell formation. Type B granules are hypothesized to be the yolk granules, as yolk granules occur in the oocytes of all archoophoran platyhelminths.

The observed increase in volume densities of the Type B granules and the lipid droplets in the laid, cleaving embryo when compared to the unlaid egg was initially perplexing. There is no indication of new synthesis of granules. A decrease in volume of the embryo following egg laying was hypothesized. Morphometric measurements of unlaid eggs and laid, developing embryos of *C. pulchra* demonstrated that the volume of the laid embryo is significantly less than that of the unlaid egg. This change in volume, which may well be a fixation artifact, probably accounts for the apparent two-fold increase in volume densities of Type B granules and lipid droplets.

To our knowledge, the only turbellarians for which the origin of eggshells has been examined ultrastructurally are the rhabdocoels *Microdalyellia fairchildi* (Bunke, 1982) and *Mesostoma ehrenbergii* (Domenici and Gremigni, 1977) and the polyclads *Pseudostylochus* sp. and *Planocera multitentaculata* (Ishida and Teshirogi, 1986; Ishida, 1989). In the rhabdocoel *Microdalyellia fairchildi*, some regions of the uterine epithelium release a vesicular secretion against which EFGs from the yolk cells are secreted, although the exact role of this secretion in eggshell formation is not clear (Bunke, 1982). Ishida and Teshirogi (1986) describe a dual origin for the eggshells of the polyclads in which an eggshell envelope is synthesized by the shell glands in the female reproductive system, and the remainder of the shell is formed following release of the EFGs from the oocyte. Ishida (1989) has demonstrated experimentally that the envelope is required for formation of the eggshell. In *C. pulchra* the accessory cells to the oocyte form a thin shell or envelope against which the contents of the Type A granules are released. Thus it appears that an exogenous layer may be required to delineate the parts of the shell formed from EFGs. The origin of the external layer varies among the three groups of turbellarians studied to date. Within the polyclads, however, the origin of the external envelope is constant in the two species examined. Only with additional studies will it be possible to know if the origin of the external layer is a useful taxonomic character.

EFGs in acoelomorphans

The EFGs of *C. pulchra* are similar to the EFGs of other turbellarians in three ways: (1) they contribute to the formation of the eggshell, (2) they have a complex morphology, and (3) the synthesis of EFGs begins prior to the synthesis of yolk granules, as in other turbellarians in which yolk granules are a product of the Golgi. The EFGs of all acoelomorphans (Acoela and Nemertodermatida) studied to date differ from the EFGs of other turbellarians in that the former lack polyphenols (see Gremigni, 1988; Smith *et al.*, 1988; Falleni and Gremigni, 1990). Another apparent difference is that the EFGs found in oocytes of acoelomorphans are smaller than those in oocytes and vitellocytes of other turbellarians. EFGs of most turbellarians are 1–2 μm in diameter, whereas the average diameter of the EFGs of *C. pulchra* is only one-quarter to one-half as large, or 0.48 μm. The EFGs of other acoelomorphans appear like those of *C. pulchra*, with diameters of approximately 0.5 μm (see Gremigni, 1988; Smith *et al.*, 1988). Falleni and Gremigni (1990) have recently suggested that the EFGs of "*Convoluta psammophyla*" fuse as they migrate centripetally to form granules 1–1.2 μm in diameter.

The species of acoels that have been examined for eggshell formation belong to two different families, assuming that the "*Convoluta psammophyla*" examined by Falleni and Gremigni (Gremigni, 1988; Falleni and Gre-

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of the laid embryo is much more constant than that of the shell of the unlaid egg. Extra-embryonic space (ES) separates the shell from the developing embryo. Bar = 1.0 μm. (C) The shell (arrow) of the embryo after the first two cleavages. Micromeres (MI) and macromeres (MA) can be distinguished, and Type B granules (B) and lipid droplets (L) occur in both types of blastomeres. The extensive extra-embryonic space (ES) is shown. Bar = 10.0 μm.

migni, 1989, 1990) is, in fact, *Paedomecynostomum psammophilum* (Family Mecynostomidae; see Beklemischev, 1957; Dörjes, 1968). Differences in our results and those of Falleni and Gremigni make a morphological characterization of an EFG within the Acoela difficult at this point. The electron-dense granules similar in size to the Type A granules that form the eggshell in "*Convoluta psammophila*" (Falleni and Gremigni, 1989, 1990) appear to differ from those in *C. pulchra* in at least two ways. First, the shell-forming granules of "*C. psammophila*" occur in clusters in the ooplasm, whereas they appear to be randomly located in the oocytes of *C. pulchra*. Second, in "*C. psammophila*" the granules marginate and fuse with one another to form granules with a diameter 1–1.2 μm. The granules of *C. pulchra* were not observed to fuse with one another except occasionally at the time of exocytosis. Certainly additional studies of eggshell formation in acoelomorphans are required.

Phylogenetic implications

Smith *et al.* (1986) suggested that the Turbellaria may be polyphyletic with three distinct lineages: (1) the Catenulida, (2) the Nemertodermatida-Acoela [=Acoelomorpha (Ehlers, 1984)], and (3) the Haplopharyngida-Macrostomida-Polycladida-Neoophora and all higher parasitic platyhelminths [=Rhabditophora (Ehlers, 1984)].

The discovery that acoels' EFGs lack polyphenols prompted examination of the composition of the EFGs in the sister group to the acoels, the Nemertodermatida (Thomas *et al.*, 1985). The oocytes of the nemertodermatid *Nemertinoides elongatus* were negative for polyphenols (Smith *et al.*, 1988). The absence of polyphenols from EFGs of the Acoelomorpha stands in sharp contrast to the presence of polyphenols in EFGs in the Rhabditophora and provides further evidence supporting phyletic distance between the Acoelomorpha and the Rhabditophora. Yet to be discovered are characters that clearly link the Acoelomorpha to the other groups (Smith *et al.*, 1982). Studies of eggshell formation in the catenulids may provide this link. There are several examples of homology linking the Catenulida and the Rhabditophora, including the ciliary rootlet system in their epidermal cells and the origin of replacement cells for the epidermis in the parenchyma (Ehlers, 1984; Smith *et al.*, 1986). Oocytes of catenulids, which have an eggshell that arises from granules within the oocyte (Borkott, 1970), have never been examined by electron microscopy. It will be interesting to examine the morphology of catenulid EFGs and to find out if they contain polyphenols. If the Catenulida do have polyphenolic EFGs, this fact would further separate the Catenulida and Rhaditophora from the Acoelomorpha. Although one should consider the possibility that the lack of polyphenols in EFGs is derived, if the catenulids have a non-polyphenolic EFGs with the same morphology as the EFGs of *C. pulchra*, the morphology of the EFG could provide a link between the Acoelomorpha and the Catenulida. A non-polyphenolic EFG could then represent a plesiomorphy for the Platyhelminthes as suggested by Falleni and Gremigni (1989, 1990).

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